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(57) Abstract

Methods for quantitating the biolcgical levels of various pharmaceuticals, particularly carbohydrate pharmaceuticals, are provided for. The methods include the step of fluorescently labeling the pharmaceutical present in a sample for analysis. The fluorescently labeled pharmaceutical may then be quantitated based on measurements of fluorescence. Additionally the fluorescently labeled pharmaceuticals may be separated from other compounds in the sample by electrophoresis. Quantitation of biological levels of pharmaceuticals is used to determine optimum dosage of pharmaceuticals.

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FLUOROPHORE - ASSISTED THERAPEUTIC DRUG MONITORING

Field of the Invention

The subject invention is in the field of biological and chemical assays, in particular the measurement of levels therapeutic agents in the body.

Background of the Invention

Chemicals have been used in the treatment of disease for thousand of years. With the advent of the modern pharmaceutical era, therapeutic chemicals have been administered in measured doses.

The biologically available levels of a pharmaceutical may vary unpredictably in accordance with a number of factors including, clearance by the kidneys, binding to various serum components, rate of absorption into the body, breakdown rate, biological activity of the breakdown products, and the like. In the last decade, it has become apparent that in order to minimize toxic effects, and maximize therapeutic effects, one must accurately measure levels of pharmacologically active chemicals. Therapeutic drug monitoring (TDM) is currently performed for more than two dozen pharmaceutical treatments. Assays on a number of pharmaceutical chemicals are available; the total number of pharmaceutical chemical assays currently available is nearly 100.

Carbohydrates are increasingly recognized as important pharmaceutical agents. Convenient assays to measure the levels of carbohydrates and similar biologicals are not readily available. In most instances the analysis of such compounds is possible only with techniques employing advanced instrumentation, such as mass spectroscopy and NMR

spectrometry. Furthermore, for some drugs, such as the anticoagulant carbohydrate drug "heparin", assays are difficult to develop because the drug is a mixture of biochemical components.

Heparin is a mixture of acidic polysaccharides derived from the proteolytic or autolytic digestion of animal connective tissue or mucosa (skin, gut, lung, or heart); for a review of heparin and its uses see Ofosu, et al., Annals of the N.Y. Acad. Sci., Vol. 556 (June 7, 1989). Heparin is assayed only on the basis of its ability to inhibit blood coagulation protein reactions. Heparin assays are typically performed by either measuring whole blood clotting times or activated clotting assays (see for example, Williams, et al., Hematology, pgs. 1256-1263, McGraw-Hill publishers (1972)) or in a research setting by measuring the activity of a specific clotting protease called anti-thrombin - III (Ofosu, et al., article by Shore, et al., ibid, pgs. 75-80).

Even when standard clotting tests are used as measurements of anticoagulant protein activities, up to 20% of patients receiving heparin experience significant bleeding. The difficulty in avoiding unwanted bleeding is because of "contaminants" in heparin or because of the variable quality of the product (Ofosu, et al., ibid, articles by Andriuoli pgs. 416-418, Lane pgs. 453-455, and Pangrazzi pgs. 468-470. Furthermore, the dosage of heparin can be related to efficiency of its use in a particular treatment situation (Ofosu, et al., articles by Sayan, et al., pgs. 476-479 and Franke, et al., pgs. 447-449) Consequently, a method to assess the qualitative purity and quantity of heparin mixtures

would be a significant improvement in the art of heparin therapy.

A highly sensitive and specific technique for separating and quantitating carbohydrates has recently been developed (Jackson, et al., Anal. Bioch. 270:705-713 (1990), U. S. patent application 07/317,480, filed February 14, 1989, U.S. patent application 07/753,196 filed August 30, 1991). This technique is called fluorophore-assisted carbohydrate electrophoresis ("FACE"). Using FACE it is possible to selectively label carbohydrates or mixtures of carbohydrates and separate the labelled carbohydrates on a matrix of polyacrylamide. The FACE technique has proven useful for the diagnosis of carbohydrate-related diseases and for quantitating small amounts of carbohydrate structures from human tissue and fluid samples, see U.S. patent application 07/676,584, filed May 7, 1991.

Summary of the Invention

One aspect of the present invention is to apply the technique of FACE to quantitate various carbohydrate and non-carbohydrate pharmaceuticals in the body so as to provide for more successful therapy.

The present invention therefore provides for the quantitative detection of a variety of fluorophore-modifiable pharmaceutical molecules, pharmaceutical carbohydrates in particular, present in tissue samples of a patient exposed to a fluorophore-modifiable pharmaceutical in the course of treatment or that which may occur accidentally. By providing for the quantitative detection of various pharmaceuticals in a patient, the present invention enables the dosage of the therapeutic carbohydrate

administered to the patient may be adjusted so as maximize the desirable effects and minimize toxicity.

This quantitation of fluorophore-modifiable pharmaceuticals is accomplished by a process comprising derivatizing a functional side group of the pharmaceutical present in a tissue sample with a fluorescent label, separating the labeled carbohydrate (preferably by electrophoresis), and identifying and quantitating the fluorophore-modified pharmaceutical, preferably by comparison with a derivatized pharmaceutical carbohydrate standard.

A preferred florescent label for modifying fluorophore-modifiable pharmaceuticals to be quantitated is 8-amino-naphthalene-1,3,6-trisulfonic acid (ANTS). The separation and quantification of the fluorophore-modified pharmaceuticals is preferably performed by polyacrylamide gel electrophoresis. The separated fluorophore-modified pharmaceutical molecules may then be visualized using an imaging system based on photoelectric detection means, such means include the use of laser-scanner photomultiplier tubes and charge coupled device (CCD) cameras. Information from the CCD camera may subsequently be stored in digital form and analyzed by various computer programs for comparing banding patterns between samples and identification standards. Gel-separated fluorophore-modified pharmaceuticals may optionally be transferred <u>in situ</u> to an immobilizing membrane; i.e., blotted, then probed with various specific-binding reagents whereby the identity and quantity of pharmaceutical molecules of interest may be determined.

Description of the Specific Embodiments

Definitions

The term "fluorophore-modifiable pharmaceutical" is defined as a pharmaceutical that is capable of being derivatized (through covalent bonds) by a fluorophore that can be employed in the technique of fluorophore-assisted carbohydrate electrophoresis. The fluorophore-assisted carbohydrate electrophoresis technique is described in detail in U.S. patent 4,874,492 and in co-pending U.S. patent application 07/317,480, filed February 14, 1989, which are herein incorporated by reference. Fluorophores that can be used for fluorophore-assisted carbohydrate electrophoresis and methods for using them are also described in U. S. patent application 07/483,043 filed February 16, 1990 and UK patent application GB/90/01448 filed September 20, 1990 and published as PCT application WO 91/05256, which are herein incorporated by reference. Fluorophores for modifying fluorophore-modifiable pharmaceuticals include 8-aminonapthalene-1,3,6-trisulphonic acid, 1-amino-6,8-disulphonic acid, 1-amino-4-naphthalene sulfonic acid, lucifer yellow, and 2-amino acridone. Fluorophore-modifiable pharmaceuticals may be derivatized using procedures described in the referenced patents and patent applications. Pharmaceuticals may be conveniently tested to determine if they are fluorophore-modifiable pharmaceuticals by attempting to fluorophore label the pharmaceuticals by the fluorophore labeling methods and labels useful for FACE. Fluorophore-modifiable pharmaceuticals typically include carbohydrates having reducing sugars and other pharmaceuticals that have aldehyde functional groups. Fluorophore-modifiable pharmaceuticals include pharmaceutical carbohydrates and pharmaceuticals that are not carbohydrates. A fluorophore-modifiable

pharmaceutical that has been labelled with a fluorophore is defined to be a "fluorophore-modified pharmaceutical."

The term "pharmaceutical carbohydrate" is defined as carbohydrates that may be used as pharmaceuticals for human or non-human animals. Pharmaceutical carbohydrates include the actual carbohydrate introduced into the body and any in vivo breakdown product or products of the carbohydrate introduced. Pharmaceutical carbohydrates include heparin, heparan sulfate, dermatan sulfate, chondroitin sulfate, hyaluronic acid, dextran, dextran sulfate, lactulose, sucralose, sucralfate, sialic acids, glucose, galactose, mannose and the like. Pharmaceutical carbohydrates may include carbohydrate antibiotics such as the aminoglycosides, beta-lactams and the like, and various phosphate and sulfate derivatives thereof. Pharmaceutical carbohydrates include polyethylene glycol (and other similar water-soluble polymer) derivatives of non-carbohydrate pharmaceuticals.

Pharmaceutical carbohydrates may be covalently joined to polypeptides or lipids, i.e., glycoconjugates, or may be independent of other molecules. Pharmaceutical carbohydrates may be monosaccharides, oligosaccharides, or polysaccharides. Pharmaceutical carbohydrates may be branched or unbranched.

The term "carbohydrate" includes molecules that are completely carbohydrate, such as monosaccharides, oligosaccharides, and polysaccharides. The term "carbohydrate" also includes glycoconjugates such as glycoproteins, glycolipids, proteoglycans, and the like.

The term "biological level", when used in reference to a pharmaceutical, is the concentration or quantity of that pharmaceutical found in a tissue sample.

The term "dosage" when use in reference to pharmaceutical refers to both the absolute amount of pharmaceutical administered at a given point in time and the frequency of administration.

General Description

The subject invention provides for the convenient measurement of biological levels of fluorophore-modifiable pharmaceuticals used for the treatment of human and animal diseases. Samples for analysis are removed from patients and subsequently treated so as to label any fluorophore-modifiable pharmaceutical present in the sample by a fluorophore suitable for use in the analytical technique known as fluorophore-assisted carbohydrate electrophoresis (FACE). Pharmaceutical molecules modified by the fluorophore label are then quantitated, preferably based on fluorescence. Typically electrophoresis is used to separate and quantitate the fluorophore-modified pharmaceuticals in a patient sample.

The subject invention possesses a number of advantages oyer conventional techniques for measuring the biological levels of pharmaceuticals, particularly carbohydrate pharmaceuticals. The present invention permits the simultaneous measurement of fluorophore-modifiable pharmaceutical quantities in multiple samples. Moreover, several pharmaceuticals in a single sample may be analyzed simultaneously. Additionally, the subject invention may be used to measure biological levels of

pharmaceuticals that are chemically uncharacterized, since the method of the subject invention does not rely on highly specific reagents. A further advantage of the subject invention is the high sensitivity of the detection system.

The method of the subject invention necessarily employs the step of fluorophore labeling fluorophore-modifiable pharmaceuticals. The fluorophore labeling of fluorophore- modifiable pharmaceuticals enables the quantitation and separation, i.e., purification, of fluorophore-modifiable pharmaceuticals. The fluorophores useful for labeling fluorophore-modifiable pharmaceuticals and the methods for performing the labeling are essentially the same as those fluorophores and methods using in the fluorophore labeling step of FACE.

One aspect of the subject invention is the measurement of the levels of fluorophore-modifiable pharmaceuticals present in a sample isolated from an individual being treated with the pharmaceutical of interest.

The quantity of fluorophore-modified pharmaceutical present in a sample may be measured by a variety of methods, these methods include the use of immunoassays specific for fluorophore-modifiable pharmaceuticals and fluorophore-modified pharmaceuticals, and the fluorescence measurement electrophoresis separation products.

Although the subject invention provides for several methods of quantitating fluorophore-modified pharmaceuticals, it is particularly preferred to apply the FACE technique to tissue samples from patients undergoing pharmaceutical treatment with

various fluorophore-modifiable pharmaceutical. The successful application of FACE to the detection of fluorophore-modifiable pharmaceuticals has a number of advantages over conventional measurement procedures for therapeutic carbohydrates, these advantages include the detection of drugs for which no specific assay is available, (possibly, for lack of antigenicity, lack of separability, lack of specifically reactive functional groups), high sensitivity, the ability to simultaneously measure several drugs, and the ability to quantitate therapeutic carbohydrates from patient samples that contain molecules that bind to the drugs of interest.

The application of fluorophore-assisted therapeutic drug monitoring of concentrations of fluorophore-modifiable pharmaceuticals in the body permits the controlled management of a variety of diseases conditions treatable by fluorophore-modifiable pharmaceuticals that require careful monitoring of biological levels in order to minimize adverse side-effects and maximize beneficial effects.

Fluorophore-modifiable pharmaceuticals may be separated by fluorophore-assisted carbohydrate electrophoresis in essentially the same way as the technique is used to separate carbohydrates. A brief description of fluorophore-assisted carbohydrate electrophoresis is given below. Fluorophore-assisted carbohydrate electrophoresis permits the electrophoretic separation of a complex mixture of carbohydrates into distinct bands on a gel or other electrophoresis separation matrix. Prior to electrophoresis, a carbohydrate mixture for analysis is treated with a fluorophore label that combines with the reducing end of the carbohydrates for analysis. The fluorophore label permits the

quantitative measurement of the labeled carbohydrates by fluorescence. The fluorophore label either is charged or coupled with a charge imparting species when the fluorophore itself is uncharged. Thus the labelling process not only fluorescently tags the carbohydrates, but imparts an ionic charge, permitting hitherto uncharged carbohydrates to migrate in an electric field. Although labeling of carbohydrates with a charged fluorophore label is preferred, carbohydrates possessing a charge strong enough to permit migration in an electrophoresis system, e.g., heparin, need not be modified by a charged fluorescent label or an uncharged florescent label coupled with a charge imparting species; instead, such charged carbohydrates may be coupled with an uncharged fluorescent label alone. Suitable fluorescent labels include 8-aminonapthalene-1,3,6-trisulphonic acid (ANTS), 1-amino-4-naphthalene sulfonic acid (ANSA), 1-aminonapthalene-6,8-disulphonic acid (ANDA), lucifer yellow, and 2-aminoacridone. A description of fluorophores suitable for use in the subject invention can be found in U. S. patent application 07/483,043 filed February 16, 1990 and UK patent application GB/90/01448 filed September 20, 1990 and published as PCT application WO 91/05256, which are herein incorporated by reference.

After the carbohydrates (or other molecules capable of being modified by the fluorophore) have been labeled, the sample is subsequently subjected to polyacrylamide gel electrophoresis, or similar separation means, in order to separate and concentrate the labeled carbohydrates into bands. The separated carbohydrates may be visualized directly (with staining) or by fluorescence under U.V. light and the banding patterns stored

photographically. Alternatively, the separated carbohydrates may be visualized by photoelectric means, including laser-scanner photomultiplier tube systems and cooled charge coupled devices (CCD). CCD's are semiconductor imaging devices that permit the sensitive detection of emitted light. CCD's and their uses are described in U.S. Patent 4,874,492 and U.S. Patent application 07/317,480, filed February 14, 1989, which are herein incorporated by reference. The image produced by the CCD may be subsequently transferred to a computer so that the band information may be analyzed with respect to intensity, mobility, standards, and the like.

When performing fluorophore-assisted carbohydrate electrophoresis in conjunction with the subject invention, electrophoretic separation typically takes place to an extent sufficient to independently resolve bands of fluorophore-modifiable pharmaceuticals. Electrophoresis may proceed past the point where some carbohydrates have been removed from the electrophoresis separation medium. Electrophoresis may be in one or in two dimensions. Two-dimensional separation of carbohydrates by fluorophore-assisted carbohydrate electrophoresis is described in U.S. patent 4,975,165, which is herein incorporated by reference. These two-dimensional electrophoresis techniques may also be applied to fluorophore-modified pharmaceuticals that are not carbohydrates.

When fluorophore-modifiable pharmaceutical compositions administered to the body comprise a mixture of structurally related molecules, such as heparin, or glycoconjugates that differ from one another with respect to the extent of glycosylation, fluorophore-assisted carbohydrate electrophoresis of

tissue samples containing the fluorophore-modifiable pharmaceutical may produce diffuse bands of labeled compounds rather than a narrow distinct band. The amount of fluorophore-modified pharmaceutical present in diffuse bands, such as the type produced by heparin, may be quantitated by determining the perimeter of the band and quantitating the florescence observed within the perimeter of the diffuse band.

Samples for analysis by fluorophore-assisted therapeutic drug monitoring may be prepared from many tissues removed from subjects. The term "tissue" includes bodily fluids as well as solid cellular masses. Tissues for analysis may contain at least one fluorophore-modifiable pharmaceutical (or the pharmaceutical breakdown product). Suitable tissues for use as samples include, blood, saliva, urine, skin, muscle, bone marrow, cerebrospinal fluid, synovial fluid, lymphatic fluid, amniotic fluid, and the like. Preferred tissues for analysis are those tissues conveniently obtained from patients, particularly preferred tissues include urine and blood. The selection of tissues for quantitation of fluorophore-modifiable pharmaceuticals by the subject invention vary in accordance with the disease and compound being analyzed. Factors affecting the choice of tissues for analysis include: the quantity of therapeutic carbohydrates present in the tissue, the quantity of background carbohydrates in the tissue, and the presence of molecules in the tissue capable of interfering with electrophoretic separation of the fluorophore-modifiable pharmaceuticals.

Fluorophore-modifiable pharmaceuticals in a sample from a patient treated with a fluorophore-

modifiable pharmaceutical may be present in a concentration that is higher than the concentration of fluorophore-modifiable pharmaceutical present in a sample from that individual prior to treatment. The term "higher" includes the presence of pharmaceuticals that are present as opposed to completely absent. Differences in fluorophore-modifiable pharmaceutical concentrations between treated and untreated individuals do not necessarily exist in all body tissues.

Fluorophore-modifiable pharmaceuticals administered to a patient may vary in concentrations with respect to different tissues. Preferred tissues for use as a source of samples for analysis by fluorophore-assisted therapeutic monitoring, are those tissues that have the highest levels of the fluorophore-modifiable pharmaceuticals of interest; however, it may also be of interest to measure the presence of pharmaceutical carbohydrates in tissues that would be not be expected to contain appreciable amount of a specific fluorophore-modifiable pharmaceutical unless the patient has been administered too large, i.e., producing unacceptable side effects, a dose that fluorophore-modifiable pharmaceutical.

Samples for analysis may require processing prior to the fluorophore labeling step in the subject method. The precise method of sample processing employed for a given assay may vary in accordance with a number of factors attributable to the choice of sample tissue and the identity of the specific fluorophore-modifiable pharmaceutical; these factors include: the concentration of the pharmaceutical, the concentration of background molecules, the presence of interfering molecules, i.e., molecules

that adversely affect the mobility or fluorophore labelling of fluorophore-modifiable pharmaceutical molecules whether the pharmaceuticals are free or bound to other molecules, and similar problems. Suitable methods for processing samples include: dialysis, to remove interfering molecules; filtration or centrifugation, to remove interfering particulates or concentrate cells; precipitation, to remove interfering molecules; and detergent solubilization, to release pharmaceutical carbohydrates from cells.

After the pharmaceuticals of interest have been separated by fluorophore-assisted carbohydrate electrophoresis, the pharmaceuticals may then be transferred in situ to an immobilizing matrix, such as a nitrocellulose or nylon membrane. The transfer may be by electroblotting or similar in situ transfer procedures. Membranes containing the pharmaceuticals may subsequently be probed with antibodies or similar specific-binding reagents so as to indicate the presence and quantity of fluorophore-modified pharmaceuticals of interest. The transfer of fluorophore-assisted carbohydrate electrophoresis separated carbohydrates onto immobilizing matrices is described in detail in U.S. patent application 07/481,367 filed February 16, 1990 and U.S. Patent 5,019,231 which is herein incorporated by reference. These membrane transfer techniques may be used with many fluorophore-modifiable pharmaceuticals, as well as carbohydrates in general.

In order to facilitate the detection and quantification of pharmaceutical carbohydrates it may be advantageous to modify the structure of some pharmaceutical carbohydrates by cleavage between carbohydrate subunits of a polysaccharide, or oligosaccharide, prior to electrophoretic separation,

or by cleaving the carbohydrate portion(s) of a glycoconjugate from the remainder of the molecule. Suitable methods of cleavage include the use of glycosylytic enzymes, either endoglycosidases or exoglycosidases. Reasons for glycosidase treatment of samples include the liberation of carbohydrates from glycoconjugates, and the generation of pharmaceutical carbohydrates derivatives that have a more convenient gel migration rate, i.e., better separation from fluorophore-labeled compounds other than the pharmaceutical carbohydrates of interest. Similarly, it may be advantageous to use a glycosyl transferase, along with a donor sugar, to structurally modify a pharmaceutical carbohydrate so as to produce a compound with a more convenient gel migration rate.

It may also be of interest to employ specific-binding reagents, such as lectins, antibodies, and the like (for a description of various antibody derivatives, see Winter and Milstein, Nature 349: 293-299 (1991)) for the purpose of quantitating and purifying fluorophore-modified pharmaceuticals. It may be possible to produce antibodies to fluorophore-modified pharmaceuticals when the parent pharmaceutical compound is not antigenic. When quantitating pharmaceutical carbohydrates, especially pharmaceutical carbohydrates that are glycoconjugates, it may be of interest to use specific binding reagents to concentrate and purify the pharmaceuticals so as to provide for more easily interpreted fluorophore-assisted carbohydrate electrophoresis results. For example, antibodies specific for the polypeptide portion of a glycoprotein with many glycoforms, and present in a tissue with a high carbohydrate background, may be used to immunoprecipitate the glycoprotein so that

the precipitated glycoprotein may then be subjected to endoglycosylase treatment to release the carbohydrate moieties prior to performing fluorophore-assisted carbohydrate electrophoresis. The purified glycoforms could thus be easily detected and quantitated in the presence of a reduced background.

In a preferred embodiment of the invention, fluorophore-modifiable pharmaceutical standards are included on the gels used to analyze pharmaceuticals in patient samples; however, the information embodied by the standards, e.g., band migration distance, band width, and intensity, may also be obtained by comparison with stored records made from fluorophore-modified pharmaceutical standards previously subjected to fluorophore-assisted carbohydrate electrophoresis under conditions similar to the conditions the samples for analysis are exposed.

Fluorophore-modifiable pharmaceutical standards may have a composition similar to that of samples for analysis in that they may contain both fluorophore-modified pharmaceuticals identical to the fluorophore-modified pharmaceuticals to be detected and background molecules with a composition similar to that found in actual samples. Alternatively, fluorophore-modifiable pharmaceutical standards may contain one or more fluorophore-modified pharmaceuticals identical to the fluorophore-modified pharmaceuticals to be detected free of background carbohydrates. Fluorophore-modifiable pharmaceutical standards for use in detecting and quantitating fluorophore-modifiable pharmaceutical that are the metabolic breakdown products of other fluorophore-modifiable pharmaceuticals may, among other methods, be produced by administering the

pharmaceutical to an animal that produces the desired break down product or by treating the pharmaceutical with enzymes participating in the breakdown process.

Fluorophore-modifiable pharmaceutical standards are fluorophore labelled, i.e., fluorophore-modified, preferably labeled prior to the labelling of the samples for analysis; however, fluorophore-modifiable pharmaceutical standards are preferably labeled concomitantly with the labeling for the samples for analysis. Furthermore, fluorophore-modifiable pharmaceutical standards are preferably of known concentrations so as to provide for a quantitative determination of the amount of a given pharmaceutical carbohydrates in the samples of analysis. Preferably, several fluorophore-modifiable pharmaceutical standards of known concentration are present on a gel so as to provide a calibration curve for more accurate determination of the quantity of pharmaceutical carbohydrates in a samples for analysis.

The subject invention also provides for kits for performing fluorophore-assisted therapeutic drug monitoring. Fluorophore-assisted therapeutic drug monitoring kits may, among other things, provide collections of reagents useful for performing fluorophore-assisted carbohydrate electrophoresis, or fluorophore-modifiable pharmaceutical standards. Kits of the subject invention enable laboratories to conveniently and reproducibly perform fluorophore-assisted therapeutic drug monitoring. Kits may include reagents for performing tests to identify one or more specific therapeutic carbohydrates. Kits may include fluorophore-modifiable pharmaceutical standards, fluorescent label, blotting materials, (such as immobilizing membranes) carbohydrate-

specific binding reagents, instructions, sample containers, polyacrylamide gel reagents, and the like. More complete kits may also include equipment for performing fluorophore-assisted carbohydrate electrophoresis, such as polyacrylamide gel apparatus, CCDs, computers, software, and the like. Reagents included in fluorophore-assisted therapeutic drug monitoring kits are preferable provided in pre-measured quantities.

In a preferred embodiment of the subject invention, band data from the gels used to separate and quantitate fluorophore-modified pharmaceuticals are read by means of a CCD and stored in a computer usable form. The image detected by the CCD, or other detection system, may be analyzed by image analysis software such as Optimas™ (Bioscan™) or similar image analysis programs. The data may be subjected to analysis by a variety of software programs. Software programs of interest include those with the ability to quantitate band intensity, measure band mobility, determine the relative molecular weight of fluorophore-modifiable pharmaceuticals forming bands, compare the standards with the samples for analysis, prepare intensity/concentration calibration curves, remove unwanted background information, and perform various forms of statistical analysis. In a preferred embodiment of the subject invention, quantitative data obtained from the fluorophore-assisted carbohydrate electrophoresis is manipulated and/or presented in electronic spreadsheet form, e.g., Lotus 1-2-3™, Microsoft Excel™.

An aspect of the subject invention is to provide for the improved management of the therapy of patients undergoing treatment with one or more fluorophore-modifiable pharmaceuticals. Improved

management of patient therapy may be achieved by monitoring the concentration of the administered pharmaceutical and/or breakdown products of the administered pharmaceutical present in patient or tissues. If the concentration of the fluorophore-modifiable pharmaceuticals as measured by fluorophore-assisted carbohydrate electrophoresis, is found to be too high (the definition of "too high" for a particular pharmaceutical carbohydrate being dependent on empirical data for that particular fluorophore-modifiable pharmaceutical carbohydrate), the amount of pharmaceutical administered may be altered by decreasing the dosage of fluorophore-modifiable pharmaceutical carbohydrate and/or the frequency of administration of the fluorophore-modifiable pharmaceutical. Similarly, if the measured concentration, preferably measured by fluorophore-assisted carbohydrate electrophoresis, of pharmaceutical is found to be too low, the amount of fluorophore-modifiable pharmaceutical administered may be altered by increasing the dosage of pharmaceutical carbohydrate, and/or frequency of administration of the pharmaceutical carbohydrate.

Measuring the amount of a fluorophore-modifiable pharmaceutical (and/or its metabolic breakdown product) by fluorophore-assisted electrophoresis may also be used as a method of diagnosing the dysfunction of an organ that is involved in the metabolic processing of fluorophore-modifiable pharmaceuticals, especially the liver or kidneys. If the concentration of a particular fluorophore-modifiable pharmaceutical (and/or its metabolic breakdown product) found in a tissue is known to be a function of how well a particular organ(s) is functioning, e.g., if the organ metabolizes or clears the pharmaceutical, then

determining the difference between the amount of a pharmaceutical carbohydrate administered and the amount found in the sample tissue, considering the amount of time that has elapsed since administration, serves to indicate how well that organ(s) is functioning. For example, a cirrhotic liver (possibly damaged as a result of a pharmaceutical carbohydrate) might not be able to degrade a particular pharmaceutical carbohydrate as fast as a healthy liver, thus blood levels of that pharmaceutical carbohydrate are found to be virtually the same at both 1 hour and 12 hours after administration, liver damage would be indicated.

The invention having been described, the following examples are offered to illustrate the subject invention by way of illustration, not by way of limitation.

EXAMPLES

I. Quantitation of Heparin Using Fluorophore-Assisted Carbohydrate Electrophoresis

Various amounts of low molecular weight heparin (Sigma Chemical Co. St. Louis, MO H271 lot 119F0921) and high molecular weight heparin (Sigma H3125 lot 90H0261) were diluted in the partially purified serum human serum to final concentrations of 1 nanomoles/ml serum.

Human serum was partially purified using a Millipore Ultra Free centrifugal concentrator, specifically to remove the high molecular weight compounds. The sample was then placed in a microcentrifuge tube and dried using a centrifugal vacuum evaporator centrifuge vacuum evaporator. To each dried sample was added 5 μl of 0.2M aminonapthalene-1,3,6-trisulphonic acid (ANTS)

solution in acetic acid/water (3:17 v/v) and 5 μl of 1.0M $NaCNBH_3$ solution in dimethyl sulfoxide (DMSO). The solution was vortex mixed, centrifuged at 10,000 g to ensure all the reactants are in the tips of the tubes, and incubated at 37°C for 15 hours. The reaction mixture was dried under a vacuum for 4 hours in a centrifuge vacuum evaporator at approximately 45°C. The reaction mixture was then dissolved in a suitable concentration of electrophoresis sample buffer.

Final concentrations of heparin, 6 to 100 picomoles, were run on gels. The individual labelled heparin bands were imaged using a CCD camera and software designed to recognize the quantitate bands. The area of florescent heparin band and the total photon count or integrated gray valve (IGV) were determined. A plot of the IGV/area valve versus heparin concentration showed linearity between 6 and 100 picomoles. The carbohydrate banding patterns could also be distinguished from one another depending on the type of heparin preparation used.

II. Quantitation of dextran sulfate in patient samples

Dextran sulfate has been used as an experimental treatment for HIV infections. Blood samples from several patients undergoing treatment with dextran sulfate are removed. Serum is isolated from the blood samples. Known quantities of dextran sulfate are added to serum so as to produce multiple quantitation standards. The serum samples and standards are partially purified using a centrifugal concentrator. The samples and standards are then placed in microcentrifuge tubes and dried. To each sample is added 5 μl of a 0.2M 2-amino acridone solution and 5 μl of 1.0M $NaCNBH_3$ in DMSO. The

solutions are mixed by vortexing, centrifuged, and incubated overnight at 37°C. The reaction mixtures are dried. The reaction mix is then resuspended in electrophoresis loading buffering. The samples and standards are then loaded on to a vertical discontinuous 8% polyacrylamide Laemli gel (lacking SDS). The gel is run at constant voltage with cooling. After the gel run is completed the individual labeled dextran sulfate bands are imaged based on their fluorescence by means of a CCD camera and software designed to recognize and quantitate bands. The quantity of fluorescence observed from the standards is plotted against the known concentration of dextran sulfate in the standards. The quantity of fluorescence observed from the labeled dextran sulfate in the patient samples is then compared with the plots obtained from the standards in order to determine the quantity of dextran sulfate present in the patient samples.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Indeed, various modifications of the above-described modes for carrying out the invention which are obvious to those skilled in the field of clinical chemistry or related fields area intended to be within the scope of the following claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Claims:

1. A method of measuring the quantity of a fluorophore-modifiable pharmaceutical in a patient sample, said method comprising the steps of,

labeling said pharmaceutical with a fluorophore, and

measuring the quantity of the labeled pharmaceutical.

2. A method according to claim 1, said method further comprising the step of, separating said pharmaceutical from other compounds in said patient sample.

3. A method according to claim 2, said method further comprising the step of, comparing the quantity of said pharmaceutical with at least one standard.

4. A method according to claim 3, wherein said fluorophore-modifiable pharmaceutical is a carbohydrate.

5. A method according to claim 4, wherein said carbohydrate is heparin.

6. A method according to claim 1, wherein said fluorophore is selected from the group consisting of 8-aminonapthalene-1,3,6-trisulphonic acid, 1-amino-6,8-disulphonic acid, 1-amino-4-naphthalene sulfonic acid, lucifer yellow, and 2-amino acridone.

7. A method according to Claim 3, wherein said standard is a breakdown product of said pharmaceutical.

8. A method according to Claim 1, said method further comprising the step of measuring the quantity of labeled pharmaceutical carbohydrates by a CCD.

9. A method of treating a patient with a fluorophore-modifiable pharmaceutical, said method comprising the steps,

administering said pharmaceutical at a first level,

removing a tissue sample from said patient,

measuring the concentration of said pharmaceutical by a method according to claim 1,

administering said pharmaceutical at a second level, wherein said second level is in part determined by said concentration measurement.

10. A method according to claim 9, wherein said fluorophore assisted electrophoresis uses a fluorophore selected from the group consisting of 8-aminonapthalene-1,3,6-trisulphonic acid, 1-amino-6,8-disulphonic acid, 1-amino-4-naphthalene sulfonic acid, lucifer yellow, and 2-amino acridone.

11. A method according to claim 9, wherein said pharmaceutical is a carbohydrate.

12. A method according to Claim 9, wherein said standard pharmaceutical is the breakdown product of said pharmaceutical.

13. A method according to Claim 9, wherein said pharmaceutical carbohydrate is selected from the group consisting of heparan sulfate, dermatan sulfate, chondroitin sulfate, hyaluronic acid, dextran, dextran sulfate, lactulose, sucralose,

sucralfate, sialic acids, glucose, galactose, mannose, aminoglycosides, and beta-lactam antibiotics.

14. A method according to Claim 9, said method further comprising the step of measuring the quantity of labeled pharmaceutical carbohydrates by a CCD.

15. A method for measuring the function of an organ in a patient, said method comprising the steps,

administering a dose of a fluorophore-modifiable pharmaceutical to said patient,

removing a tissue sample from said patient at a point in time after the administering of said carbohydrate,

measuring the concentration of said pharmaceutical in said sample by then method according to claim 1,

comparing the measured concentration of said pharmaceutical with the concentration of said pharmaceutical that would be expected to be present if said tissue sample was removed from a healthy subject having been administered said pharmaceutical at said point in time prior to the removal of said tissue sample.

16. A method according to claim 15, wherein said pharmaceutical is a carbohydrate.

17. A kit for performing fluorescence assisted therapeutic pharmaceutical carbohydrate drug monitoring, said kit comprising a fluorophore-modifiable standard.

18. A kit according to Claim 11, wherein said kit further comprises, a fluorophore label.

19. A method of treating a patient for a disease by administering a fluorophore-modifiable pharmaceutical said method comprising the steps:

administering said pharmaceutical at first dosage,

removing a tissue sample from said patient,

measuring the quantity of said pharmaceutical in said sample by the method according to claim 1,

determining if said quantity is effective for the treatment of said disease,

adjusting said dosage to as to provide for the effective treatment of said disease.

20. A method according to claim 19, wherein said pharmaceutical is a fluorophore-modifiable carbohydrate.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/10061

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]
According to International Patent Classification (IPC) or to both National Classification and IPC
Int.Cl. 5 G01N33/52; G01N31/22; G01N33/66

II. FIELDS SEARCHED	
Minimum Documentation Searched[7]	
Classification System	Classification Symbols
Int.Cl. 5	G01N
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]	

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO,A,9 105 256 (ASTROSCAN LIMITED) 18 April 1991 cited in the application see claims; example 3	1-8,17, 18
X	US,A,5 019 231 (B. K. BRANDLEY ET AL.) 28 May 1991 cited in the application see the whole document	1-8

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° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION	
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19 MARCH 1993	20. 04. 93
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	GRIFFITH G.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	CHEMICAL ABSTRACTS, vol. 90, 12 March 1979, Columbus, Ohio, US; abstract no. 86155, J. M. MENTER ET AL. 'Kinetics of fluorescence fading of acridine orange-heparin complexes in solution' page 511 ;column 2 ; see abstract & PHOTOCHEM. PHOTOBIOL. vol. 27, no. 5, 1978, pages 629 - 633	1
X	CHEMICAL ABSTRACTS, vol. 102, no. 14, 8 April 1985, Columbus, Ohio, US; abstract no. 119748, M. M. AYAD ET AL. 'Spectrofluorometric microdetermination of imidazoline derivatives using 1-dimethylaminonaphthalene-5-sulfonyl chloride' page 410 ;column 2 ; see abstract & ANALYST (LONDON) vol. 109, no. 11, 1984, pages 1431 - 1434	1
X	JOURNAL OF PHARMACEUTICAL SCIENCES vol. 63, no. 9, September 1979, WASHINGTON US page 1097 C. H. CHI ET AL. 'Sensitive fluorescence assay for d,l-methadone'	1
X	DE,A,3 912 046 (CARNEGIE-MELLON UNIVERSITY) 15 March 1990 see claims 1,8	1
A	WO,A,8 810 422 (ASTROSCAN LIMITED) 29 December 1988 cited in the application	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 92/ 10061

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Claims searched completely: 1-8, 15-18.
Claims not searched: 9-14, 19-20 see PCT-Rule 39.1 (iv):
Method of treatment of human or animal body by therapy.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9210061
SA 67439

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 19/03/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9105256	18-04-91	AU-A-	6400790	28-04-91
		EP-A-	0494178	15-07-92
		JP-T-	5500563	04-02-93
US-A-5019231	28-05-91	AU-A-	7034991	03-09-91
		EP-A-	0515379	02-12-92
		WO-A-	9112276	22-08-91
		US-A-	5094731	10-03-92
DE-A-3912046	15-03-90	JP-A-	2191674	27-07-90
WO-A-8810422	29-12-88	EP-A-	0318559	07-06-89
		GB-A-	2215836	27-09-89
		JP-T-	1503807	21-12-89
		US-A-	5104508	14-04-92

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82